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Diversity of transport mechanisms: common structural principles

Arnold J.M. Driessen, Barry P. Rosen and Wil N. Konings

Traditionally, prokaryotic solute transport systems are classified into major groups based on the energetic requirement of the transport process. These include the secondary transporters that are driven by a proton or sodium motive force, and the ATP-binding cassette (ABC) primary transporters, which use the hydrolysis of ATP to fuel transport. These transporters are specified by entirely different architectures of polypeptides. Recently, transport systems have been discovered that are composed of combinations of distinct functional modules of both secondary and ABC transporters. These findings indicate that during evolution the combination of integral membrane transport proteins with either a periplasmic solute-binding protein or a cytosolic ATPase, or both, have resulted in distinct classes of transporters with unique architectures and properties.

AROUND 1960, Leon Heppel discovered that osmotically shocked *Escherichia coli* had drastically reduced transport activities for a variety of solutes, including amino acids, ions, sugars and vitamins[1]. Subsequent studies revealed that this reduced transport activity was caused by a loss of periplasmic solute-binding proteins (SBPs). These proteins are part of a transport system that is composed of two identical or homologous cytoplasmic membrane domains and two identical or homologous peripheral membrane-associated ATP-binding domains (Fig. 1a)[2]. In Gram-negative bacteria, SBPs freely diffuse in the periplasm, whereas in Gram-positive bacteria, they are often anchored to the cytoplasmic membrane by a lipid moiety, and some are even fused to the membrane domain[3]. In Archaea, SBPs are anchored to the membrane by one or more transmembrane segments (TMS)[4]. Apart from the structural similarity, SBP-dependent transport systems have several functional characteristics in common: they are high-affinity (usually in the submicromolar range) ATP-driven transporters that function in a unidirectional manner to drive the accumulation of solutes against high concentration gradients. The genes coding for the structural components of these transport systems are usually organized in a locus. Because an ATP-binding cassette is highly conserved in this class of transport proteins, they are also termed ABC transporters[5,6]. This family of transport systems also includes a multitude of transporters that lack an SBP: for example, transporters that participate in the excretion of a variety of compounds such as antibiotics, drugs and oligosaccharides[5]. The driving force for solute accumulation via these systems is supplied by the hydrolysis of ATP, and they are called primary transporters.

Another major class of transport systems comprises the ‘secondary’ transporters[7]. A large group of these secondary transporters belong to the major facilitator superfamily (MFS)[8]. Typically, they consist of a single integral membrane protein that spans the membrane 12 times (Fig. 1b). The 12 transmembrane segments are interconnected by large cytosolic loops and short external loops. These systems predominantly catalyse solute: H^+/Na^+ symport or antiport driven by the proton or sodium motive force (pmf/smf). The transport affinity for the solute is

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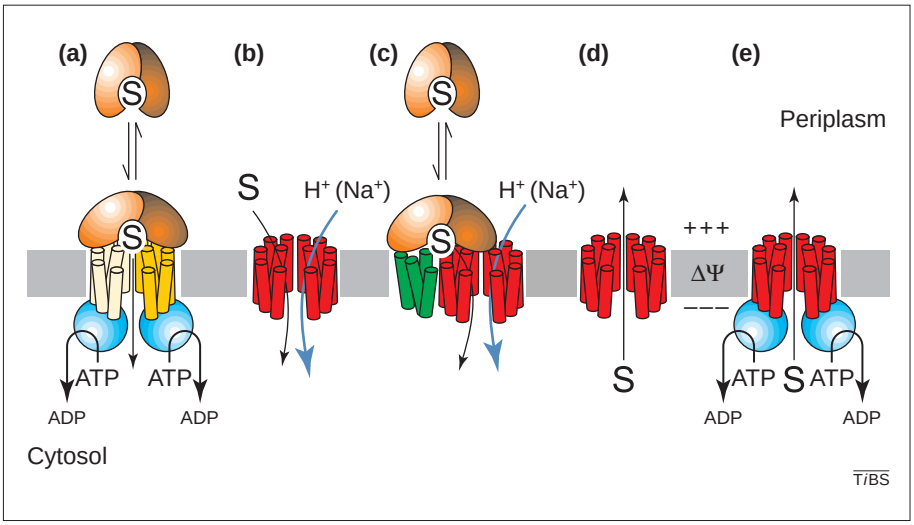

Figure 1

Domain structure of bacterial transport systems. **(a)** Solute-binding protein (SBP)-dependent ATP-driven primary transport systems. A periplasmic SBP binds to the solute (S) and donates it to the multisubunit transporter domain that consists of two identical or homologous integral membrane proteins and two identical or homologous ATP-binding domains. ATP hydrolysis drives the unidirectional uptake of the solute. **(b)** Secondary uptake transporters. Uptake of a solute is coupled to the cotransport of protons (or Na^+ ions) and therefore driven by the pmf/smf. Transport is reversible and usually mediated by a single integral membrane protein. **(c)** SBP-dependent secondary transporters. A periplasmic SBP binds to the solute and transfers it to a membrane domain that consists of a secondary carrier-like domain and a small integral membrane protein. Uptake is coupled to the cotransport of protons (or Na^+ ions) and driven by the pmf/smf. Moreover, transport is unidirectional. **(d)** Secondary excretion transporters. ArsB is a secondary arsenite transporter that catalyses anion extrusion coupled to the transmembrane electrical potential, $\Delta\Psi$, which is positive on the outside. The solute represents arsenate. **(e)** ATP-driven excretion transport systems. ArsA is an ATPase, which associates with ArsB yielding a complex that functions as an anion-translocating ATPase. Note that the SBP is monomeric and typically consists of two domains, which, upon binding of the solute, close to yield an occluded state of the bound solute. In the open state, the SBP releases the solute to the transporter.

usually in the micromolar range and transport is often reversible; that is, these systems catalyse exchange and efflux. Depending on the physiology of the microorganism or the environmental conditions, either of these transport systems could be prominently present as the main mechanism to accumulate solutes. In a bacterial cell, ~10% of the genes encode for transport proteins (see complete list of prokaryotic genomes on the TIGR database: http://www.tigr.org/). On the one hand, in *E. coli*, transport proteins belonging to the MFS and the ABC transporters are present in roughly equal numbers, and, for most amino acids, there is a transporter present belonging to each class. On the other hand, in pathogens like *Haemophilus influenzae*, *Mycoplasma genitalium* or the thermophile *Thermatoga maritima*, the predominant mechanism of transport is SBP dependent and ATP coupled. In the yeast *Saccharomyces cerevisiae*, there are approximately five times as many members of the MFS as ABC transporters.

In recent years, novel transporters have been identified that not only share energetic and catalytic characteristics of both classes but that even combine distinct modules of either class to yield a unique architecture or energy requirement, or both.

Secondary solute-binding-protein-dependent transporters: variation on a theme or a new class?

Rhodobacter sphaeroides is a free-living purple photosynthetic bacterium, which has been widely used as a model for the study of photosynthesis, nitrogen fixation and the regulation of gene expression in response to environmental factors. It can grow chemo- or photoheterotropically on a wide variety of carbon sources and relies mainly on SBP-dependent transport systems for the uptake of nutrients (T. Abee, PhD thesis, University of Groningen, 1989). Glutamate transport in this microorganism is catalysed by both an SBP-dependent and ATP-coupled transporter, and a specific glutamate transporter that functions via an entirely different mechanism. From a combination of *in vivo* and *in vitro* studies[9], this transport system for glutamate has been identified and characterized. It involves a SBP that does not interact with an ABC transporter but with a secondary transporter. The driving force for uptake is supplied by the pmf/smf and not by ATP. These conclusions are drawn from the following observations.

First, cells that are defective in the SBP-dependent ATP-driven transporter for glutamate and glutamine[10] are still able to accumulate glutamate. Uncouplers and ionophores that collapse the pmf inhibit this residual glutamate transport activity. The system does not seem to use ATP for transport and is not inhibited by vanadate, a phosphate analog that acts as a strong inhibitor of plasma membrane P-type ATPases and ABC transporters[9]. By contrast, glutamate uptake via the SBP-dependent ATP-driven transporter is inhibited by vanadate and is insensitive to uncouplers[10].

Second, the residual glutamate transport activity in these cells is osmotic-shock sensitive and can be restored in spheroplasts by the addition of osmotic shock fluid or by purified glutamate-binding protein[9]. This SBP is monospecific for glutamate and binds the substrate with high affinity. It cannot substitute for the glutamate/glutamine-binding protein associated with the SBP-dependent ATP-driven transporter.

Third, *in vitro* uptake of glutamate can be observed in isolated membrane vesicles of *Rb. sphaeroides* cells that lack the SBP transport system, only when a pmf/smf is generated by electron transport. However, this transport activity is strictly dependent on the addition of purified monospecific glutamate-binding protein[9]. Also, in these membrane vesicles, glutamate uptake is sensitive to uncouplers and ionophores and is not inhibited by vanadate.

Another interesting aspect of glutamate transport into cells and membrane vesicles of *Rb. sphaeroides* is that transport is Na^+ dependent, with half-maximal saturation at 25 mM Na^+ (Ref. 9). Because of this low affinity for Na^+, it is not known whether Na^+ is co-transported or whether it acts as an allosteric regulator. The glutamate-transport system does not catalyse exchange or efflux, a property that is typical for SBP-dependent transport systems. *In vivo* studies using uncouplers and vanadate suggest that the C_4-dicarboxylate transporters of *Rb. capsulatus*[10] and *Rb. sphaeroides*[9] are other examples of such a novel system. However, in these cases no *in vitro* studies have been carried out, which could support the proposed mechanism of transport. Thus, this

novel bacterial transport system does not seem to belong to either one of the known classes of transport mechanisms but appears to share properties of both.

The question arises whether these transporters are mere hybrids of the known classes of transporters or whether they represent a new family of transport proteins. The genes encoding for the C_4-dicarboxylate transporter of *Rb. capsulatus* have been cloned and are encoded by the *dct* locus[11,12]. The *dct* locus contains three structural genes, *dctP*, *dctQ* and *dctM*, which are essential for transport. DctP is a typical SBP (Refs 13,14), whereas DctQ and DtcM are integral membrane proteins with molecular weights of 25 and 47 kDa, respectively. The *dct* locus does not contain a gene encoding an ABC protein, consistent with the notion that this locus encodes a novel SBP-dependent secondary transporter[11]. The biochemical demonstration of a unique transport mechanism now allows us to assign DctP, DctQ and DctM as components of this hitherto unrecognized class of SBP-dependent secondary transporters. The term TRAP-T (for tripartite ATP-independent periplasmic transporters) has been introduced by Kelly and co-workers[11], but this name does not cover the entire family, as some transporters seem to use only one large integral membrane domain instead of two. We therefore prefer to use the term SBP-dependent secondary transporters to separate them from the SBP-dependent ABC transporters. The DtcP, DctQ and DctM proteins are homologous to the products of previously unidentified ORFs in a large number of Gram-negative bacteria, including *E. coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Bordella pertussis, Pseudomonas aeruginosa* and the cyanobacterium *Synechocystis*[11,15,16]. Members of this family have also been found in the archaea *Aeropyrum pernix* and *Archaeoglobus fulgidus*, and the Gram-positive bacterium *Enterococcus faecalis*[16], suggesting that they are present in all major prokaryotic subfamilies. Typically, these putative transporters consist of one SBP and two integral membrane proteins. However, in *H. influenza* (which contains three systems that belong to this class), in one of the systems, the DctQ and DctM homologues are fused into a single large integral membrane protein with a molecular mass of ~70 kDa. Although there is significant homology to the C_4-dicarboxylate transporter of *Rb. capsulatus*, the nature of the solute that is transported by these putative transport systems is unknown. So far, the biochemical evidence is restricted to glutamate and C_4-dicarboxylates, both anionic organic solutes. The specific need for SBP-dependent secondary transporters is essentially unknown. If the substrates are indeed transported as anions, it could be difficult for cells to maintain a large concentration gradient across the membrane as the transmembrane electrical potential ($\Delta\Psi$), negative on the inside, will counteract any uptake. Imposing unidirectionality to the system could overcome this problem, but it is a mystery why SBP-dependent secondary transporters (and ABC transporters) function unidirectionally. Curiously, neither SBP-dependent ABC nor secondary transporters have been found in eukaryotes.

The integral membrane domain of the SBP-dependent secondary transporters has a unique organization. Hydropathy analysis[17] and application of the 'positive-inside' rules of von Heijne[18] on an alignment of all sequences of the family of DctQ and DctM homologues predict the presence of four and 12 potential TMS, respectively. The hydropathy pattern of DctM, which contains 12 TMS with a central hydrophilic cytosolic loop, shows a striking similarity to that of secondary transporters. At the primary sequence level, however, DctM and homologous proteins are very dissimilar to secondary transporters or to the membrane domains of SBP-dependent ABC transporters. Moreover, they all lack the conserved $EAAAx_3Gx_9IxLP$ motif that identifies the membrane domain of SBP-dependent transport systems and is thought to be involved in the interaction with the ATP-binding subunit[19]. They also lack the typical Walker consensus distinctions, Gx_4GKT and $Rx_{4–12}h_4D$ (h, hydrophobic amino acid), that are part of the ATP-binding site of ABC transporters[5,6]. Unique signature sequences have been defined for the family of SBP-dependent secondary active transporters[15,16], but their function is unknown.

The presence of two integral membrane subunits in the family of SBP-dependent secondary transporters suggests a two-domain structure. This larger subunit could be responsible for the actual transport reaction and energy coupling to the pmf/smf, whereas the smaller subunit might be needed to interact with the SBP (Fig. 1c). The latter function is normally not contained in secondary transporters. The SBP that belongs to these transporters functionally resemble the ones that interact with ABC transporters[12,13], but they are not interchangeable[9,10].

The double life of the arsenite efflux transporter: a secondary or primary transporter?

For the arsenite transporter, there is not a singular mode of energy coupling,

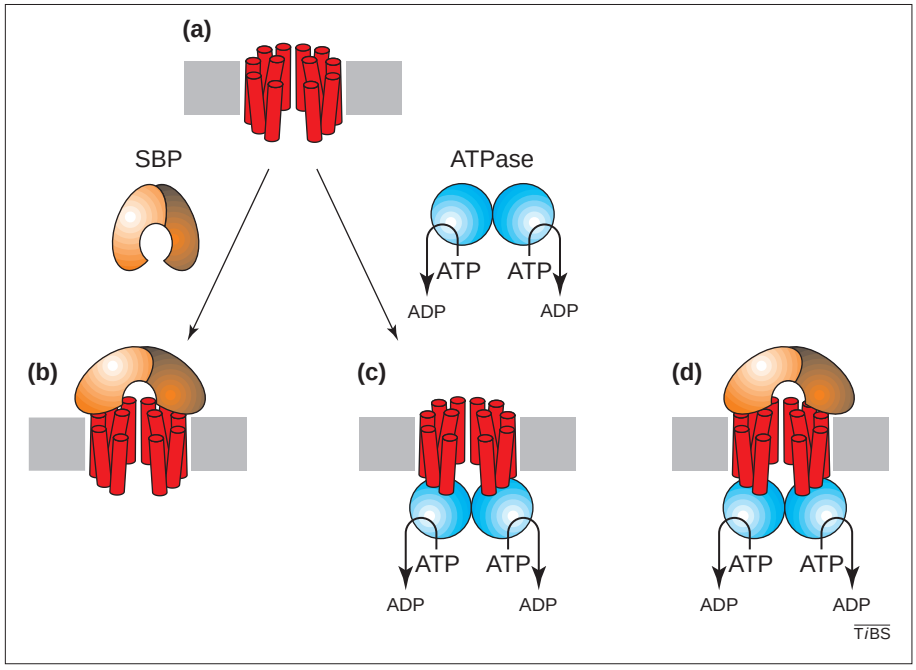

Figure 2

Hypothetical model of the evolution of transport systems based on their structural similarities. The model proposes that during evolution, ancestors of the secondary transport proteins **(a)** have associated with either soluble substrate-binding proteins or ATPase to yield solute-binding protein (SBP)-dependent secondary transporters **(b)** or ATP-driven transporters **(c)**. These systems might be the progenitors for the SBP-dependent ATP-driven transporters **(d)**. The SBP is indicated as a monomeric, two-domain protein.

but, depending on the polypeptide architecture, the system could either function as a primary or secondary transporter. This transporter provides bacterial resistance to salts of the metalloids arsenic and antimony in Gram-negative and Gram-positive bacteria[20]. This system is encoded by the arsenical resistance (*ars*) operon that generally constitutes three genes, *arsRBC*. The *arsR* gene product is an As(III)/Sb(III)-responsive transcriptional repressor, and the *arsC* gene product is an arsenate reductase that reduces arsenate [As(V)] to arsenite [As(III)], expanding the range of resistance to include both ionic species. The *arsB* gene product is a 45-kDa membrane protein that is sufficient to confer resistance to the metalloid oxyanions arsenite and antimonite. It is a transport protein that catalyses extrusion of the toxic arsenite or antimonite anion. ArsB has 12 TMS, with a topology similar to that of many secondary transporters[21]. From a combination of *in vivo* and *in vitro* studies, which are outlined below, it is clear that arsenite transport catalysed by ArsB requires only the pmf and not ATP, suggesting that ArsB is a secondary transporter that extrudes the oxyanion out of cells[22,23].

In cells expressing *arsB*, transport requires the pmf and is inhibited by uncouplers[22]. For these studies, an *unc* strain of *E. coli* lacking the H^+-translocating ATPase that catalyses the equilibrium between ATP and the pmf was used. On the one hand, energy-depleted cells produce only chemical energy (e.g. ATP) when given glucose in the presence of an inhibitor of respiration (e.g. cyanide). On the other hand, these cells will generate a pmf only when given a respiratory substrate such as succinate. With this protocol, intracellular conditions can be established in which either chemical energy or a pmf is available for transport.

In vitro accumulation of $^{73}AsO_2^{-1}$ can be observed in everted membrane vesicles[23]. Everted membrane vesicles have an orientation opposite to that in intact cells. Cells extrude arsenite, and everted membrane vesicles accumulate the anion. Only a pmf generated by NADH respiration supports ArsB-mediated transport in membrane vesicles. This transport is sensitive to the addition of uncouplers, which further supports the contention that arsenite uptake in these everted membrane vesicles is coupled to the pmf. Although the relative contribution of the transmembrane pH gradient and electrical potential has not been determined, it is assumed that ArsB is an anion uniporter (Fig. 1d).

Although the majority of *ars* operons have only three genes, *arsRBC*, some have five genes, *arsRDABC*, possibly as a result of a relatively recent insertion of the *arsDA* genes[20]. The *arsD* gene product is a second transcriptional repressor, but the *arsA* gene product is a 63-kDa ATPase that associates with ArsB to improve its properties dramatically. ArsB provides moderate resistance to metalloid salts, whereas ArsA and ArsB together confer substantially higher resistance. By associating with the ArsA ATPase, the ArsB secondary transporter (Fig. 1d) is converted into a primary ATP-coupled arsenite transporter (Fig. 1e).

A number of lines of investigation have led to this conclusion. First, ArsA and ArsB are found as a complex in isolated *E. coli* membranes[24]. Using an *in vitro* binding assay, purified ArsA protein binds to membranes containing ArsB in a saturable manner. The ArsAB complex is quite stable, dissociating only in the presence of chaotropic agents. Second, when the *in vivo* energetic experiments were performed in cells expressing both the *arsA* and *arsB* genes, quite different results were obtained, which clearly demonstrate that the ArsAB complex is an obligatorily ATP-coupled primary transporter[22]. In an *unc* strain of *E. coli* that expressed both ArsA and ArsB, succinate no longer supports arsenite extrusion, and glucose-coupled transport is insensitive to uncouplers and respiratory chain inhibitors. These results indicate that ArsAB-catalysed extrusion is coupled to chemical energy and not to the pmf (i.e. electrochemical energy). Third, although *in vivo* experiments do not identify the direct donor of chemical energy, direct coupling to ATP to $^{73}AsO_2^{-1}$ transport was accomplished using everted membrane vesicles of cells of the *unc* strain expressing both ArsA and ArsB (Ref. 25). Neither other nucleoside triphosphates nor the nonhydrolyzable ATP analogue ATPγS substitute for ATP. In membrane vesicles from the *unc* strain, arsenite transport is not driven by oxidation of lactate or NADH, and ATP-dependent transport of $^{73}AsO_2^{-1}$ by the ArsAB complex is insensitive to uncouplers. These data show that the pmf is neither necessary nor sufficient for the ArsAB transporter. Arsenite accumulation in membrane vesicles is neither inhibited by vanadate nor by azide, an inhibitor of F-type ATPases.

The exciting conclusion that can be drawn from these results is that transport of arsenite via the integral membrane protein ArsB can be energized by a pmf or by ATP hydrolysis depending on the association with ArsA; ArsB alone functions only as a secondary transporter (Fig. 1d), whereas the ArsAB complex is an obligatorily ATP-coupled primary transporter (Fig. 1e). Of note is the fact that both modes are physiological – bacteria with the three-gene operon use the pmf to extrude arsenite; those with the five-gene operon use ATP. Both allow survival in moderate concentrations of arsenic salts, but an ArsAB ATPase confers an evolutionary advantage to organisms exposed to high levels of arsenic salts. Because a primary transporter is capable of forming higher concentration gradients than secondary transporters, the ArsAB system reduces the intracellular concentration of metalloid ion to lower levels than can be realized by ArsB alone.

How did these classes of transporters evolve?

The findings of SBP-dependent secondary transporters and of ABC transporters, which are converted to secondary transporters upon removal of the ATPase domains, indicate that different combinations of an integral membrane transporter protein, a periplasmic SBP and a cytosolic ATPase have been formed during evolution. These different combinations have led to different classes of transporters with different architectures and properties.

In the SBP-dependent secondary transporters, a novel small membrane protein, which is not homologous to proteins with known functions, is fused to or separated from a membrane domain of which the hydropathy profile resembles a secondary transporter. Their membrane domain structure suggests that at least part of the transporter must have evolved from an ancestral secondary transporter, which has acquired the ability to interact with an SBP (Fig. 2). Presumably because of this interaction, the transporter must have somehow lost the ability to catalyse exchange and efflux. With the identification of orthologs in a wide variety of prokaryotes including pathogens, it will be a challenge to analyse this new class of transporters with respect to the functions of the putative domains and compare their properties with the classical systems that have been studied in detail for the last decades. Also, it will be

necessary to purify these transporters and functionally reconstitute them into proteoliposomes to demonstrate unambiguously the energetic mechanism of transport.

The arsenical resistance system is encoded by a five-gene operon, which might have evolved from a three-gene operon by acquisition of an *arsDA* operon, and the original function of ArsD could have been the regulation of expression of ArsA (Ref. 20). The function of the progenitor of ArsA is unclear, but it was probably a cytosolic protein with ATPase activity. Is the ArsAB unique in having evolved from the association of a secondary transporter with a soluble ATPase? The system is unique in the sense that ArsB can function physiologically either as a secondary transporter or as a subunit of a primary transporter, sometimes even in the same strain of bacteria. For example, *E. coli* has an *arsRBC* operon in its chromosome but frequently carries five gene operons on plasmids. It is interesting to speculate that other primary transporters might have evolved from secondary transporters. Two examples of such systems are the F_oF_1 H^+-translocating ATPases[26] and the ABC transporters[27]. The F_oF_1 is composed of the F_1 catalytic sector, which is a soluble ATPase in the absence of the F_o sector. In the absence of the F_1, the F_o is a H^+-conducting complex that transports protons into the cell in response to a pmf. In contrast to ArsB, however, F_o does not appear to have a physiological role by itself, although it might have evolved as an independent proton transporter that became the membrane component of a proton pump by association with the ancestor of the F_1 (Fig. 2).

Many of the bacterial ABC transporters that catalyse uptake of solutes are primarily multisubunit systems[5,27]. The ATPase subunit is associated with membrane subunits. For example, in the maltose permease[28], MalK is the catalytic subunit and can be purified as a soluble ATPase, and MalF and MalG are separate membrane subunits that form the sugar translocation pathway. Although they do not function as secondary transporters in the absence of MalK, they might have evolved from proteins that did (Fig. 2). In conclusion, other transport ATPases might have originated from the independent evolution of the catalytic and translocation modules as soluble ATPases and secondary transporters, with subsequent and graduate association to produce a functional transport system.

Conclusions

From the evidence presented above, it appears that during evolution secondary transport proteins have associated with either SBP, cytosolic ATPase (possible ancestors of F_1) domains, or both SBP and ATPase domains (Fig. 2). The acquisition of binding proteins resulted in transporters with higher substrate affinities, whereas the acquisition of the ATPase domains constructed transporters with increased translocation power (higher capacity). This unique combination of functional units and further diversification during evolution has led to a number of recognizable classes of transporters, which differ in architecture and energy requirements.

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